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High expression of Livin serves as a predictive indicator for parotid gland tumors

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Abstract. – OBJECTIVE: The aim of this study was to explore the expression of Livin in benign and malignant parotid gland tumors. We also investigate the role of Livin in the occurrence and progression of parotid gland tumors.

PATIENTS AND METHODS: Livin expression in 30 cases of normal parotid gland tissues, 40 cases of benign parotid gland tumors and 60 cases of malignant parotid gland tumors was detected by immunohistochemistry. The correlation between Livin expression and pathological characteristics of patients with parotid gland tumors was analyzed. The differentially expressed Livin in normal parotid gland tissues and malignant parotid gland tumors was determined by Western blot and quantitative Real Time-Polymerase Chain Reaction (qRT-PCR), respectively.

RESULTS: Livin expression was unable to be detected in normal parotid gland tissues. The positive rate of Livin expression in benign and malignant parotid gland tumors was 17.50% and 71.67%, respectively ($p<0.05$). Livin expression was correlated to malignant level, clinical stage and tumor diameter in 60 cases of malignant parotid gland tumors ($p<0.05$). However, the positive rate of Livin expression was not correlated to lymph node metastasis, age and sex of patients with parotid gland tumors ($p>0.05$).

CONCLUSIONS: Livin expression is closely related to the pathological progression of parotid gland tumors, which may serve as a hallmark in diagnosis, treatment and prognosis of patients with parotid gland tumors.

Key Words
Livin, Parotid gland tumor, Immunohistochemistry.

Introduction

Parotid gland tumors are common tumors in the oral and maxillofacial region, accounting for about 80% of parotid tumors, and the proportion of benign and malignant tumor is about 5:1[1]. Pathological subtypes of parotid gland tumors are complicated, and their biological behaviors vary a lot, which are key factors influencing recovery and life quality of affected patients. Currently, surgical resection in the main treatment for parotid tumors. Appropriate radiotherapy and chemotherapy are applied for those high-malignant parotid tumors that are unable to be operated[2,3]. However, surgical treatment poses a great influence on the face appearance, leading to local scars, facial paralysis, and deafness syndrome. Therefore, researches on novel treatment for parotid tumors are required[4,5].

The occurrence and development of malignant parotid gland tumors are complicated, involving abnormal proliferation, differentiation and apoptosis of parotid gland tumor cells. Imbalanced apoptosis and proliferation are one of the crucial mechanisms leading to malignant parotid gland tumor[6,7]. Weakened apoptosis and enhanced proliferation of tumors cells are affected by the external factors[8]. Hence, apoptosis-related genes are greatly involved in the malignant progression of parotid gland tumors, requiring for in-depth study. At present, apoptosis has been well studied in tumor research. Livin, as an inhibitor of apoptosis, is a new member of the inhibitor of apoptosis protein (IAP) family. In 2000, Livin was screened out for the first time from the human genome database for the homologous sequence of the IAP family[9,10]. Currently, IAP family members include eight species, namely XIAP, cIAPI, cIAP2, NAIP, Livin, apollon, ILP-2, and survivin[11]. As a newly discovered member of the IAP family, highly expressed Livin in a variety of tumor cells would result in the chemotherapy resistance[12]. In this investigation, Livin expression in benign and malignant parotid gland tumors, as well as normal parotid gland tissues was de-

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tected by immunohistochemistry and molecular biology. The direct correlation between Livin expression and clinical data of patients with parotid gland tumors was further explored.

Patients and Methods

Patients

A total of 100 patients with parotid gland tumors treated in The Affiliated Yantai Yuhuangding Hospital of Qingdao University between January 2017 and January 2018 were enrolled, including 43 males and 57 females (aged 18-87 years, mean age = 42.4 years). Parotid gland tumors were surgically resected and collected as experimental groups. Based on the pathological classification, 25 cases were parotid pleomorphic adenoma, 15 were other benign parotid tumors, and 60 were malignant parotid tumors. Additionally, 30 cases of normal parotid tissue around the tumor edge were used as a control group. None of the enrolled patients were treated with radiotherapy and chemotherapy before surgery. The TNM (Tumor Node Metastasis) staging and histological grades were collected from their medical records. The TNM staging and the histopathological classification were evaluated based on the UICC (2002) standard and the WHO 2005 standard, respectively. This research was approved by the Ethics Committee of The Affiliated Yantai Yuhuangding Hospital of Qingdao University. Signed written informed consents were obtained from all participants before the study.

Immunohistochemistry

All samples were fixed in 10% neutral formaldehyde solution, paraffin-embedded and sliced into 4-μm slides. Immunohistochemistry was performed using the Envision method. The slide was dewaxed, hydrated, and incubated with 3% H_2O_2 at room temperature. After antigenic heat repair, incubation of Livin antibody was performed overnight at 4°C. At the other day, tissue samples were incubated with IgG for 2 h at 37°C. DAB (diaminobenzidine) (Solarbio, Beijing, China) was used for color development, and then slides were sealed with neutral resin and air dried. Three randomly selected fields in each sample were selected for the analysis of Livin-positive rate by Image-pro-plus immunohistochemical image software. Livin expression was evaluated based on the staining density (-, no Livin-positive cells; +, Livin-positive cells<25%; ++, 25%-50%; +++, >50%).

Western Blot

Parotid gland tissues were allocated and the supernatant was used to quantify the protein. Electrophoretic and transmembrane were carried out. Non-specific sites of the membrane were blocked in 5% skim milk for 90 min at room temperature. Membranes were incubated at 4°C overnight with primary antibody. The corresponding secondary antibody labeled by peroxidase was added for incubation at room temperature for 2 h. Images were obtained by continuous exposures through the UVP chemiluminescence imaging system. The final results were semiquantitative analyzed based on the optical density of the target protein relative to β-actin.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

The total RNA was extracted by TRIzol method (Invitrogen, Carlsbad, CA, USA). RNA content was determined by the acid protease apparatus and then diluted with Diethyl pyrocarbonate (DEPC) water (Beyotime, Shanghai, China) to the concentration of 0.5 μg/μL. Complementary Deoxyribose Nucleic Acid (cDNA) was synthesized according to TaKaRa RNA PCR Kit (AMV) Ver.3.0 kit (Otsu, Shiga, Japan). Reverse transcription products were used as templates to prepare PCR reaction system. Primer sequences were shown as follows: β-actin forward: 5'-AATGAGCGGTTCCGATGC-3', reverse: 5'-GGAAGGTGGACAGTGAGGC-3'; Livin forward: 5'-GTCAGTTCCTGCTCCGGTCAA-3', reverse: 5'-GGGCACTTTCAGACTGGACCTC-3'.

Statistical Analysis

Statistical analysis was carried out by Statistical Product and Service Solutions (SPSS) 19.0 statistical software (IBM, Armonk, NY, USA). Data were expressed by $\bar{x}\pm s$. The χ^2-test was used in comparison between two different groups. $p<0.05$ was considered statistically significant.

Results

Positive Rate of Livin Expression in Parotid Gland Tumor

We did not observe Livin expression in normal parotid gland tissues. On the contrary, Livin was mainly distributed in the cytoplasm and rarely distributed in the nucleus of parotid gland tumors. Livin was differentially expressed in different pathological types of parotid gland tumors (Table I and Figure 1A-1I). Among 40 benign pa-

Table I. Overall expression of Livin in normal or malignancy parotid tissues.

Groups	n	Livin expression				Positive rate (%)
		–	+	++	+++	
Normal parotid tissue	30	30	0	0	0	0
Parotid pleomorphic adenoma	25	19	4	2	0	24.00
Other benign parotid tumors	15	14	1	0	0	6.67
Parotid mucinous epidermoid carcinoma	12	2	5	6	1	83.33
Parotid acinar cell carcinoma	12	6	4	2	0	50.00
Salivary adenoid cystic carcinoma	11	3	5	1	2	72.73
Parotid squamous cell carcinoma	8	2	1	4	1	75.00
Parotid lymphoepithelial carcinoma	8	3	2	3	0	62.50
Epithelial-myoepithelial carcinoma	5	0	1	0	4	100.00
Myoepithelial carcinoma	4	1	3	0	0	75.00

rotid gland tumors we collected, Livin was positively expressed in 7 tumors (17.50%). Besides, 43/60 (71.67) malignant parotid gland tumors showed positive expression of Livin. A significant difference in the positive rate of Livin expression was found between benign and malignant parotid gland tumors (Table II). The positive rate of Livin expression is higher in malignant parotid gland tumors than that of benign parotid gland tumors and normal parotid gland tissues.

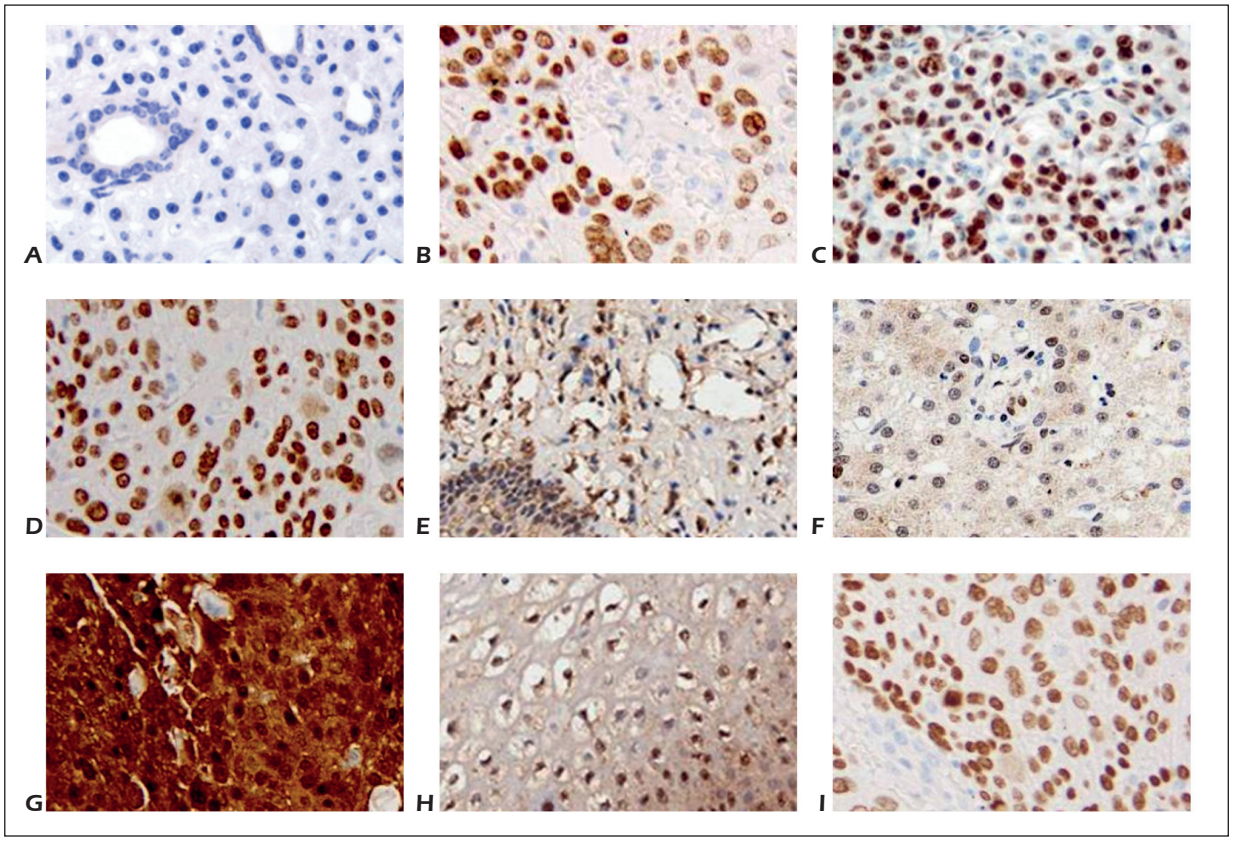

Figure 1. Livin expression in normal parotid gland tissues and parotid gland tumors detected by immunohistochemistry (magnification 200×). **A**, Negative expression of Livin in normal parotid gland tissues. **B**, Positive expression of Livin in pleomorphic adenoma. **C**, Positive expression of Livin in myoepithelial carcinoma. **D**, Positive expression of Livin in lymphoepithelial carcinoma. **E**, Positive expression of Livin in squamous cell carcinoma. **F**, Positive expression of Livin in epithelial-myoepithelial carcinoma. **G**, Positive expression of Livin in acinar cell carcinoma. **H**, Positive expression of Livin in adenoid cystic carcinoma. **I**, Positive expression of Livin in mucoepidermoid carcinoma.

Table II. Expression of Livin in benign parotid tumors and malignant tumors.

Groups	n	Livin expression		Positive rate (%)
		Positive	Negative	
Benign tumor of parotid gland	40	33	7	17.50
Parotid malignant tumor	60	17	43	71.67

Livin Was Highly Expressed in Parotid Gland Tumor

We extracted protein and mRNA samples from 10 cases of normal parotid gland tissues and parotid gland tumors. Western blot results showed higher protein level of Livin in parotid gland tumors than that of normal parotid gland tissues (Figure 2A). QRT-PCR data showed similar results as higher mRNA level of Livin in parotid gland tumors than that of normal parotid gland tissues (Figure 2B).

Positive Expression of Livin Indicated Poor Performance in Malignant Parotid Gland Tumors

Chi-square test showed that the biological behaviors, clinical stage and tumor diameter were correlated to the positive expression of Livin in 60 cases of malignant parotid gland tumors. The positive rate of Livin expression in the high-malignant tumors was 92% (23/25), which was 57.1% in low-grade malignant tumors (20/35), and the difference was statistically significant ($p<0.01$). The positive rate of Livin expression in parotid gland tumors with clinical stage I-II and III-IV was 48.4% (18/31) and 86.2% (25/29), respectively ($p<0.05$). According to the tumor size, the positive rate of Livin expression in parotid gland tumors with diameter $\leq$ 2 cm was 52.4% (11/21), which was 82.1% (32/39) in those with diameter > 2 cm ($p<0.05$). However, the positive rate of Livin expression was not correlated to lymph node metastasis, age and sex of patients with parotid gland tumors ($p>0.05$, Table III). These data suggested that positive expression of Livin in parotid gland tumors predicted poor tumor progression and Livin may serve as a biomarker for evaluating the development of parotid gland tumors.

Discussion

Parotid gland tumor is a common salivary gland tumor in the oral and maxillofacial region. Pathological type and histological morphology of parotid gland tumor are complex. Usually, benign parotid gland tumors experience a better prognosis and lower recurrence rate after surgical resection than those malignant tumors, except for pleomorphic adenoma of the parotid. However, therapeutic efficacy and long-term survival of patients with malignant parotid gland tumor vary a lot due to biological performances of tumors with different pathological types[13]. The specific pathogenesis of parotid gland tumor has not been fully elucidated. Surgical resection combined with chemotherapy and radiotherapy is still the preferred option for patients with parotid gland tumor. Unfortunately, the prognosis and long-term survival of these patients are far away from satisfactory[14,15]. In-depth studies on the molecular mechanism of parotid gland tumor are urgently

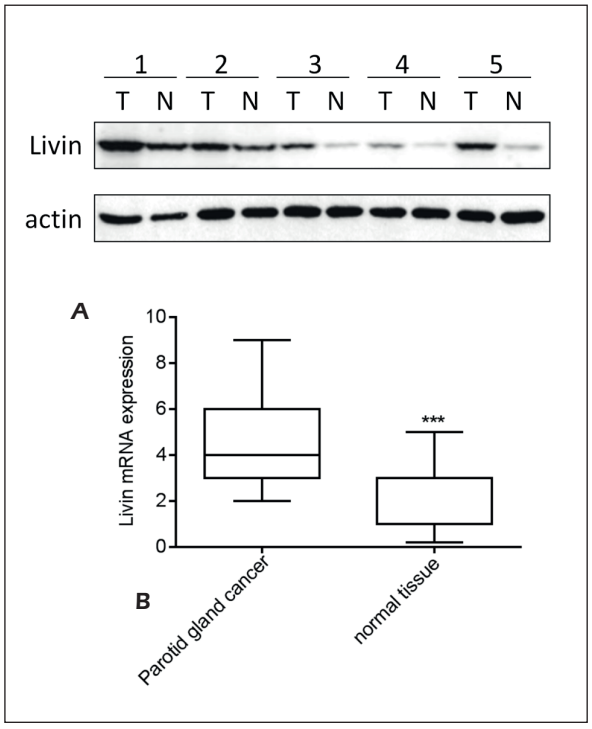

Figure 2. Protein and mRNA levels of Livin increased in malignant parotid gland tumors. A, Western blot was used to detect protein expression of Livin in 5 representative parotid gland tissues and tumor tissues. B, QRT-PCR was used to detect mRNA expression of Livin in 5 representative parotid gland tissues and tumor tissues.

Table III. Relationship between expression of Livin and clinical biological characteristics.

Clinical parameters	n	Livin expression		χ^2	p
		Positive	Negative		
Age					
<45	24	9	0.041	0.533	
≥45	19	8			
Gender					
Female	30	12	0.004	0.605	
Male	13	5			
TNM staging					
I and II	18	13	5.844	0.015	
III and IV	25	4			
Lymph node metastasis					
Yes	9	3	0.082	0.541	
No	34	14			
Tumor diameter (cm)					
≤2	11	10	5.918	0.017	
>2	32	7			
Biological behavior					
High grade malignancy	23	2	8.726	0.003	
Low grade malignancy	20	15			

needed, which may contribute to providing new directions in target treatment and individualized treatment for the parotid gland tumor.

Livin is the newest member of the IAP family. Livin is located on chromosome 20, q13.3, with a total length of about 46 Kb. It contains 7 exons and 6 introns. Livin protein contains one BIR structure at the N-terminus and one RING structure at the C-terminus. There were two mRNA subtypes of Livin transcripts because of different splicing modes, namely Livinα and Livinβ, which encode 298 and 280 amino acids, respectively. Livinα and Livinβ present different anti-apoptotic effects and distributions[16]. Livin is expressed in embryonic tissues and exerts a high expression in various tissues during fetal development, which may be related to growth and development. In most terminal tissues of normal adults (except for the placenta), Livin is barely expressed. However, Livin is highly expressed in most malignancies, such as melanoma, breast cancer, cervical cancer, colon cancer, bladder cancer, prostate cancer, leukemia, lymphoma, esophageal cancer, and lung cancer[17-19].

Livin is mainly distributed in the nucleus, whereas cytoplasmic Livin is expressed in the form of filaments. Livin is capable of inhibiting apoptosis by downregulating the activities of caspase-3 and caspase-9[20]. In addition, Livin is involved in the TNF-mediated pathway and exerts anti-apoptotic effects by interacting with NF-κB[21]. Traditional tumor treatments are based on early surgery, accompanied by radiotherapy, chemotherapy, immunotherapy, hyperthermia, and in our country traditional Chinese medicine treatment. Current studies pointed out that Livin knockdown can greatly enhance the sensitivities to radiotherapy and chemotherapy, and induce antigen-antibody immune response[22].

As a tumor antigen, antibodies that specifically recognize Livin in the peripheral blood stimulate natural killer cells to infiltrate melanoma tissues[23]. It is concluded that Livin exerts a tumor-suppressor effect by gene intervention and immune regulation, contributing to novel tumor treatments.

In this study, 60 cases of parotid gland tumors and 30 normal glandular tissues around the tumor were collected for detecting Livin expression by immunohistochemistry. We found that Livin was not expressed in normal parotid gland tissues. The positive expression of Livin in pleomorphic adenoma of parotid was significantly higher than other types of benign parotid gland tumors, which may be explained by its invasive characteristics. Besides, we found that Livin was differentially expressed in benign and malignant parotid gland tumors. Livin expression is positively correlated to the malignant level of parotid gland tumors, suggesting its potential value in early diagnosis of patients with parotid gland tumors. Since Livin expression is unable to be detected in normal glandular tissues, it may be utilized as target therapy of malignant parotid gland tumors in the future. We also analyzed the correlation between Livin expression and basic characteristics of patients with parotid gland tumor. Our results found that Livin

expression was positively correlated to tumor size, TNM stage and malignant level, but not correlated to age, sex and lymph node metastasis of patients with parotid gland tumors. The detection of Livin expression contributes to the early diagnosis and treatment of parotid gland tumors.

Conclusions

We found that the Livin expression is closely related to the pathological progression of parotid gland tumors, which may serve as a diagnostic, therapeutic and prognostic hallmark in patients with parotid gland tumors.

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Conflict of Interests

The authors declare that they have no conflict of interest.

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